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Potentiation of antitumor efficacy of paclitaxel by recombinant tumor necrosis factor-α

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We studied the combination of tumor necrosis factor (TNF) and paclitaxel. Our aim was to determine whether TNF increases the antitumor efficacy of paclitaxel and if so whether the increase is mediated through the enhancement of apoptosis induction by paclitaxel. Mice bearing 6 mm MCa-K or MCa-4 mammary carcinomas, OCa-I ovarian carcinomas, or HCa-I hepatocarcinomas in their legs were treated with TNF, paclitaxel or their combination. TNF was administered i.p. daily at a dose of 10 μg per mouse for 7 days; paclitaxel at a dose of 40 mg/kg per mouse was given as a single i.v. injection 1 h before the second dose of TNF. Tumor growth delay was used as the endpoint of tumor response to the treatments. The results showed that the combination was either additive or supraadditive; supra-additive action occurred in three of the four tumors tested. The enhancement factors (EFs) were 1.24 for MCa-K, 1.53 for Mca-4, 1.0 for OCa-I and 2.17 for HCa-I. Histological analysis of treated MCa-K tumors revealed that TNF alone did not induce apoptosis of tumor cells, but in the combination it enhanced the apoptotic response to paclitaxel. Thus, TNF increased the antitumor efficacy of paclitaxel by enhancing cellular sensitivity to paclitaxel's induction of apoptosis. The results imply that the combination of TNF and paclitaxel has potential as a treatment for cancer.

Key words: Apoptosis, murine tumors, paclitaxel, tumor necrosis factor.

Introduction

Tumor necrosis factor-α (TNF), a polypeptide mediator of the cellular immune response, has a wide range of biological activities, including direct killing of cancer cells *in vitro*[1,2] and antitumor effects *in vivo*.[3,4] Its *in vivo* antitumor effects are either direct, resulting from cytotoxic action on tumor cells, or indirect, through the modulation of antitumor immune rejection responses or damage to tumor vasculature, or both direct and indirect. TNF acts both as an immune modulator and as a mediator of monocyte cytotoxicity induced by other lymphokines and monokines.[5,6] Regarding its effects on the tumor vasculature, TNF can be both cytostatic and cytotoxic for endothelial cells,[7] augment adherence of neutrophils and lymphocytes to endothelium,[8,9] and promote clot formation.[10] Because of these pleiotropic activities, TNF holds promise for use in combination with other anticancer agents.

The therapeutic potential of TNF in combination with radiation or chemotherapy has been investigated in *in vitro* cell[11–16] and *in vivo* tumor[14,17–20] systems. When combined with radiation, TNF produced either additive or synergistic cell killing of *in vitro* cultured tumor cells.[15,16] The synergism was attributed to increased production of free radicals, which mediate oxidative cellular damage.[21,22] Our group was the first to report the *in vivo* therapeutic potential of TNF when combined with radiation: a recombinant human TNF (rHuTNF) augmented the response of a murine mammary carcinoma to ionizing radiation, as demonstrated by increases in tumor growth delay and rate of tumor cure.[17,18] These observations were confirmed by Huang *et al.*,[20] who found that TNF greatly increased radiocurability of human glioblastoma xenografts in SCID mice. Recently, a gene therapy approach using TNF in combination with radiation was tested:[23] the TNF gene was linked to a radiation-inducible promotor and the construct was transfected into tumor cells. These cells, grown as xenografts in nude mice, released TNF upon exposure to radiation, which resulted in an enhanced radioresponse of the xenografts.

TNF has also been reported to display additive or synergistic action when combined with chemotherapeutic drugs both *in vitro*[11–14] and *in vivo*.[14,19]

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The drugs include adriamycin, actinomycin D and bleomycin. No information is available on the therapeutic action of TNF combined with paclitaxel, however. This chemotherapeutic drug is highly effective against many experimental animal[24,25] and human[26,27] tumors. The cytotoxic action of paclitaxel has been attributed to its ability to bind to cellular tubulin structures, whereby it interferes with tubulin polymer formation and induces tubular bundling.[28] These changes block cell division, resulting in the arrest of cells in the G_2 and M phases of the cell cycle.[28,29] In addition to inducing mitotic arrest, paclitaxel has been reported to induce apoptotic cell death.[25,30,31] In one study apoptosis correlated with the antitumor efficacy of paclitaxel.[25] Paclitaxel was found to induce TNF gene expression in and TNF release by murine peritoneal macrophages,[32] but these actions appeared unrelated to the effects of paclitaxel on microtubule assembly and its cytotoxicity *in vitro*.[33] These observations do, however, suggest that TNF might play a role in the *in vivo* antitumor efficacy of paclitaxel.

Our study, reported here, was designed to test whether administration of TNF to mice bearing syngeneic tumors increases the antitumor efficacy of paclitaxel and if so whether the potentiation was mediated by enhancing the apoptotic response of tumor cells to paclitaxel. The antitumor efficacy of the TNF plus paclitaxel combination was determined using four different murine tumors.

Materials and methods

Mice

Inbred C3Hf/Kam male mice bred and maintained in our own specific-pathogen-free mouse colony were used. Mice were 11–13 weeks old at the beginning of the experiment. They were housed five per cage.

TNF

Vials containing rHuTNF with a specific activity of 5.62×10^4 U/mg and concentration of 1 mg/ml were kindly supplied from Hanhyo Institute of Technology (Shihung, Korea). Before use, the drug was diluted with phosphate-buffered saline to the desired concentration. For the experiments, a daily dose of 10 μg rHuTNF per mouse was injected i.p. on seven consecutive days.

Paclitaxel

Paclitaxel was kindly supplied by Dr John Whisnant (Baker Norton Pharmaceutical, Miami, FL) in dry powder form. A 5× stock solution was prepared in a 50:50 mixture of Cremophor EL (Sigma, St Louis, MO) and dehydrated ethanol. This mixture was dissolved by sonicating it for 30 min. Stock solutions were diluted 1:4 with sterile physiological saline and injected i.v. within 15 min of preparation. A single dose of 40 mg/kg paclitaxel was injected per mouse.

Tumors

Four tumors syngeneic to C3Hf/Kam mice were used: the mammary carcinomas MCa-K and MCa-4, the ovarian carcinoma OCa-I, and the hepatocarcinoma HCa-I. Single-cell suspensions were obtained by enzymatic digestion of tumor tissue using 0.025% trypsin and DNase.[34] The viability of the tumor cells, assessed by Trypan blue dye exclusion and phase contrast microscopy, was more than 90%. To generate solid tumors, 5×10^5 cells were injected s.c. in the right hind thighs of mice.

Assays of tumor response to the treatments

Tumor growth delay was used to determine tumor response to the treatments. When the tumors grew to 6 mm in diameter, the mice were randomly assigned to a treatment group. After treatment, the tumors were measured at 2–3 day intervals until they reached 14 mm, at which time the mice were killed. For each tumor, average tumor diameter was obtained from three orthogonal measurements with calipers.

The effect of the treatments on tumor regrowth was expressed as absolute growth delay (AGD). The AGD was defined as the time in days for the tumors to reach 10 mm in treated mice minus the mean time to reach 10 mm in the untreated control group. The enhancement factor (EF) was calculated by dividing normalized tumor growth delay (NGD) by AGD. The NGD was defined as the time in days for tumors to reach 10 mm in mice treated by the combination treatment minus the time in days to reach 10 mm in mice treated by TNF only. The mean (± SE) growth delay was computed for each group consisting of eight to 10 mice.

Histological determination of mitosis and apoptotic indices

At different time intervals ranging from 1 to 48 h after the treatment, mice were killed by cervical dislocation and their tumors immediately removed and placed in neutral buffered formalin. Three mice were used for each time point. For each histological type, tumors were also removed from four untreated mice. The tissues were embedded in paraffin blocks and 4 μm sections were cut from these and stained with hematoxylin and eosin (H&E).

The morphological features used to identify mitosis and apoptosis in murine tumors have been previously described and illustrated.[31] Briefly, five fields of non-necrotic areas were selected randomly across each tumor section, and in each field apoptotic bodies and cells in mitosis were expressed as a percentage based on the scoring of 1500 nuclei (2000 nuclei for untreated controls) at each time interval after treatment.

Results

The investigations focused on MCa-K, a tumor sensitive to TNF treatment that was previously used in our studies on the combination of TNF and local tumor irradiation.[17,18] To determine the response of MCa-K to paclitaxel, mice bearing 6 mm tumors in their hind thighs were given i.v. paclitaxel, at a dose of 40 mg/kg, and tumor growth delay was measured. Figure 1 shows that paclitaxel delayed tumor growth significantly. To investigate the effect of paclitaxel at the cellular level, MCa-K tumors were histologically analyzed for mitotic arrest and apoptosis at times ranging from 1 to 48 h after administration of paclitaxel. As shown in Figure 2, tumors responded by a rapid mitotic arrest that peaked at 9 h, at which time 23.2 ± 0.7% of tumor cells were mitotic; the percentage of mitotic cells returned to the baseline level (1.4 ± 0.1%) around 24 h after paclitaxel. Apoptosis developed, peaked and returned to the background level later than did mitotic arrest. Apoptosis peaked 24 h after paclitaxel, at which time 13.5 ± 2.5% of tumor cells were apoptotic. The apoptotic response returned to the background level of 0.7 ± 0.4% 36 h after paclitaxel. We have previously reported that it is primarily mitotically arrested cells that undergo apoptotic cell death after paclitaxel.[31]

The histological appearance of a MCa-K tumor that was not treated (Figure 3) and that of one that was treated with paclitaxel 24 h earlier (Figure 4) are shown. While untreated tumors displayed rare mitotic figures and apoptotic cells, paclitaxel-treated tumors exhibited an abundance of apoptotic cells and some mitotically arrested cells, the latter showing the characteristic coronal appearance of condensed chromatin.

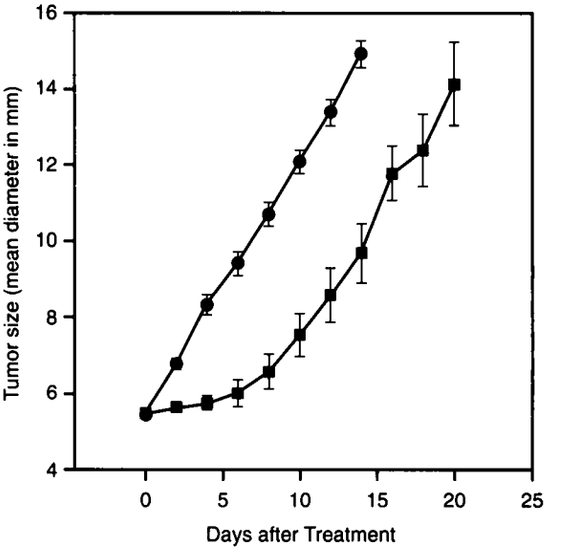

Figure 1. Effect of paclitaxel on tumor growth rate of MCa-K tumors. Tumor-bearing mice were treated with 40 mg/kg paclitaxel and tumor growth was measured. Untreated animals (●); paclitaxel-treated animals (■). Vertical bars are SE of mean.

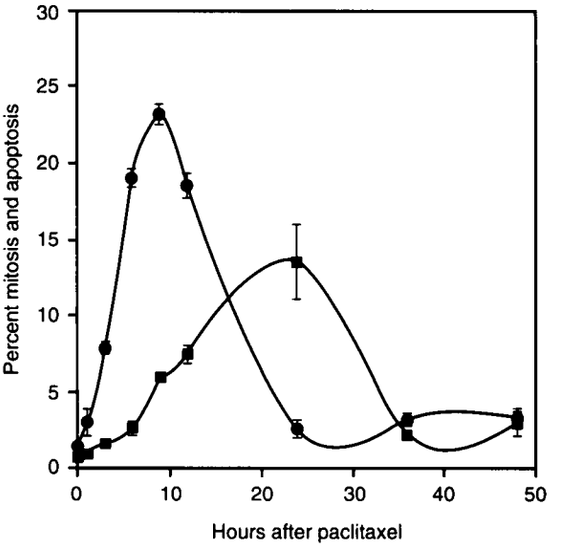

Figure 2. Percentage of mitosis (●) and apoptosis (■) in MCa-K tumors after 40 mg/kg paclitaxel treatment. Histological sections were scored for mitosis and apoptosis 0–48 h after treatment. Vertical bars are SE of mean.

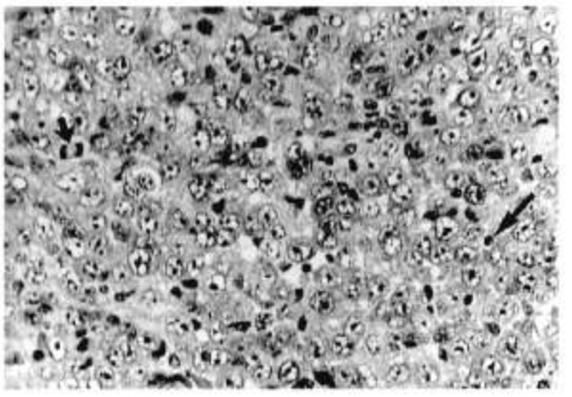

Figure 3. Histology of MCa-K tumor, untreated. Mitotic figures (short arrow) and apoptotic cells (long arrow) are rarely seen.

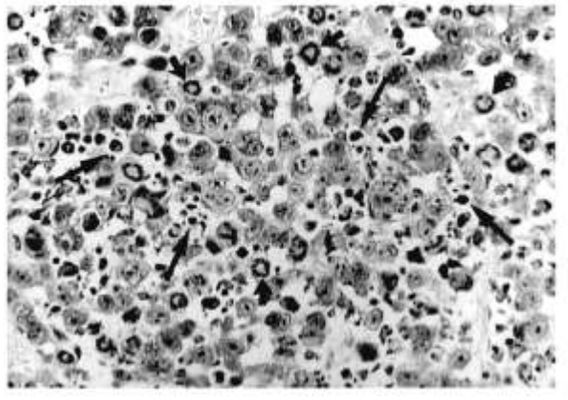

Figure 4. Histology of MCa-K tumor 24 h after paclitaxel. There is an abundance of apoptotic cells (long arrows) and some mitotically arrested cells (short arrows), the latter showing the characteristic coronal appearance of condensed chromatin.

To determine whether antitumor efficacy of paclitaxel could be further improved by TNF, 6 mm tumors were treated with TNF, paclitaxel or a combination of the two agents. TNF, at a dose of 10 μg per injection, was administered i.p. daily for 7 days, a schedule previously shown to be effective in improving radiocurability of this tumor.[17] Paclitaxel, 40 mg/kg i.v., was given once 1 h before the second dose of TNF. The results, presented in Table 1, show that TNF was effective in delaying MCa-K tumor growth, but its effect was less than that of paclitaxel. When TNF was combined with paclitaxel, the AGD was more than the additive effects of individual treatments. TNF augmented the antitumor action of paclitaxel by an EF of 1.24.

To determine whether TNF's potentiation of paclitaxel-induced tumor growth delay was mediated by enhancing the cellular effects of paclitaxel, tumors treated with TNF, paclitaxel or both were histologically analyzed for mitotic arrest and apoptosis. A single injection of 10 μg TNF or daily injections of the same dose of TNF for 7 days had no significant influence on either mitotic or apoptotic index determined 4 or 24 h after the treatment was completed (Figure 5). However, when combined with paclitaxel, a single dose of TNF given either 24 h before or 1 h after paclitaxel affected both paclitaxel-induced mitotic arrest and apoptosis determined at 4 or 24 h after the last treatment (Figure 6). Paclitaxel-induced mitotic arrest was significantly decreased by TNF when assayed 4 h after paclitaxel but was only slightly increased when assayed 24 h after paclitaxel. In contrast, TNF increased paclitaxel-induced apoptosis assayed 24 h after paclitaxel. TNF was effective when given either before or after paclitaxel. These results suggest that TNF augments the antitumor action of paclitaxel by rendering tumor cells more sensitive to induction of apoptosis.

To assess whether TNF can augment the antitumor activity of paclitaxel in other murine tumors, mice bearing 6 mm MCa-4, OCa-I or HCa-I tumors were treated with TNF, paclitaxel or the combination of the two agents using the same treatment schedule used for the treatment of MCa-K tumors. As shown in Table 2, TNF alone was only slightly effective against MCa-4 and HCa-I tumors, whereas it was highly effective against OCa-I. Paclitaxel alone was also the most effective in OCa-I, strongly

Table 1. Antitumor efficacy of TNF, paclitaxel or TNF plus paclitaxel against MCa-K murine tumor

Treatment[a]	Days to grow to 10 mm (mean ± SE)	AGD[b]	NGD[c]	EF[d]
No treatment	6.9 ± 0.5			
TNF	11.9 ± 1.2	5		
Paclitaxel	14.9 ± 1.2	8		
TNF + paclitaxel	21.8 ± 1.4	14.9	9.9	1.24

[a]Mice bearing 6 mm tumors in the right hind thighs were treated with TNF, paclitaxel or the combination of the two agents. TNF, at a dose of 10 μg per mouse, was administered i.p. daily for 7 days, and paclitaxel, at a dose of 40 mg/kg, was given as a single i.v. injection. When the two agents were combined, paclitaxel was given 1 h before the second dose of TNF.
[b]AGD is defined as the time in days for the tumors to reach 10 mm in a treated mouse minus the mean time to reach 10 mm in the untreated control group.
[c]NGD is defined as the time in days for tumors to reach 10 mm in mice treated by the combination treatment minus the time in days to reach 10 mm in mice treated by TNF only.
[d]EF is obtained by dividing NGD by AGD.

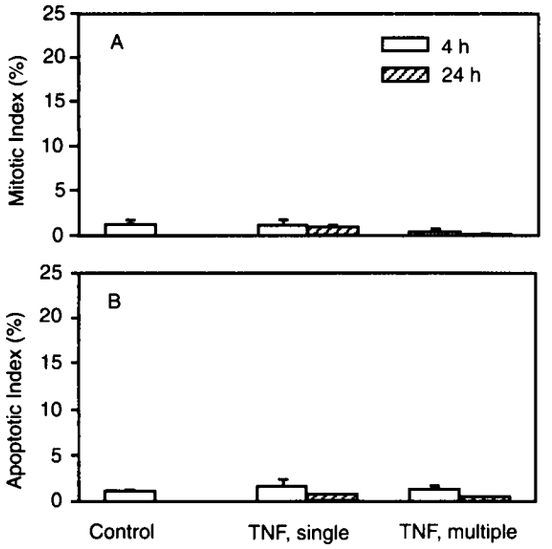

Figure 5. Mitotic index (A) and apoptotic index (B) in MCa-K tumors after TNF injection. Mice bearing 6 mm tumors were treated with TNF, at a dose of 10 μg per mouse, in single or multiple (7 days) i.p. injections. The percentage of apoptosis or mitosis was scored from histological sections made from control or treated tumors, in which samples were taken at 4 h (opened bar) and 24 h (shaded bar) after the last treatment. Vertical bars are SEM.

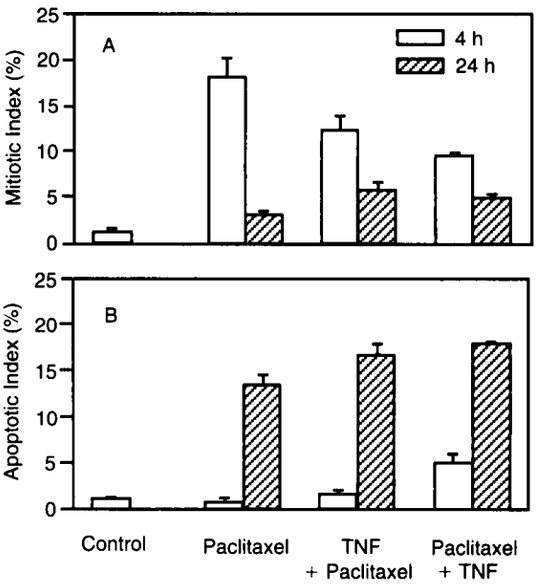

Figure 6. Mitotic index (A) and apoptotic index (B) in MCa-K tumors after treatment with the combinations of TNF with paclitaxel. Mice bearing 6 mm tumors were treated with paclitaxel or the combination of TNF and paclitaxel. Paclitaxel, at a dose of 40 mg/kg, was given as a single i.v. injection and TNF, at a dose of 10 μg per mouse, was administered as a single i.p. injection either 24 h before or 1 h after TNF injection. The percentage of apoptosis or mitosis was scored from histological sections made from control or treated tumors, in which samples were taken at 4 h (opened bar) and 24 h (shaded bar) after the last treatment. Vertical bars are SEM.

effective against MCa-4 and only slightly effective against HCa-I. When TNF and paclitaxel were combined, the effect was more than additive against MCa-4 and HCa-I, where the EFs were 1.53 and 2.17, respectively. Interestingly, the OCa-I tumor, which was the most responsive to both TNF and paclitaxel as single treatments, showed no enhanced response when the two agents were combined.

Discussion

The results presented here show that TNF acted either additively or supra-additively when combined with paclitaxel in the treatment of murine tumors. The supra-additive action occurred in three of four tumors tested. Our data also showed that an enhanced apoptotic response was an underlying mechanism of the supraadditive effect.

TNF used here was a newly developed recombinant form of TNF that was shown to be highly effective in inhibiting the growth of Meth-A fibrosarcomas in BALB/c mice.[35] It was effective against all four tumors used in the present study but to a variable degree. The most responsive was the OCa-I and the least responsive was the HCa-I tumor. This TNF was somewhat more effective in delaying the growth of murine tumors than that used in our earlier studies.[17,18] The reasons for the variability in response to TNF is not known, but the causes may be multiple since TNF can act either directly[1,2] or indirectly. It indirectly affects tumor growth through immune effector mechanisms[5,6] or by compromising tumor microcirculation.[7–10] Recently there have been a number of reports that TNF can induce apoptosis both *in vitro*[36–38] and *in vivo*.[39,40] Our present study tested whether TNF affected growth of the MCa-K tumor by inducing apoptosis. Neither single nor multiple administrations of TNF were effective in inducing apoptosis (Figure 5), suggesting that mechanisms other than apoptosis were involved in the effect of TNF against MCa-K tumor. A possibility that induction of apoptosis played a role in antitumor activity of TNF against the other three tumors used in this study was not tested.

Compared with TNF, paclitaxel exerted a stronger antitumor effect. Paclitaxel was the most effective

Table 2. Antitumor efficacy of TNF, paclitaxel or TNF plus paclitaxel against MCa-4, OCa-I and HCa-I murine tumors

Tumors	Treatment[a]	Days to grow to 10 mm (mean ± SE)	AGD[b]	NGD[c]	EF[d]
MCa-4	no treatment	11.5 ± 1.1			
	TNF	12.5 ± 2.2	1		
	paclitaxel	17.3 ± 3	5.8		
	TNF + paclitaxel	21.4 ± 1.9	9.9	8.9	1.53
OCa-I	no treatment	13.1 ± 0.4			
	TNF	19.6 ± 1.4	6.5		
	paclitaxel	32.8 ± 1.3	19.7		
	TNF + paclitaxel	39.3 ± 2.4	26.2	19.7	1
HCa-I	no treatment	5.5 ± 0.4			
	TNF	6.5 ± 0.3	1		
	paclitaxel	7.3 ± 0.5	1.8		
	TNF + paclitaxel	10.4 ± 0.4	4.9	3.9	2.17

[a]Mice bearing 6 mm tumors in the right hind thighs were treated with TNF, paclitaxel or the combination of the two agents. TNF, at a dose of 10 μg per mouse, was administered i.p. daily for 7 days and paclitaxel, at a dose of 40 mg/kg, was given as a single i.v. injection. When the two agents were combined, paclitaxel was given 1 h before the second dose of TNF.
[b]AGD is defined as the time in days for the tumors to reach 10 mm in a treated mouse minus the mean time to reach 10 mm in the untreated control group.
[c]NGD is defined as the time in days for tumors to reach 10 mm in mice treated by the combination treatment minus the time in days to reach 10 mm in mice treated by TNF only.
[d]Enhancement factor is obtained by dividing NGD by AGD.

against OCa-I, where it produced an AGD of 19.7 days, was strongly effective against MCa-K and MCa-4 (AGDs were 8 and 5.8 days, respectively), and was only slightly effective against HCa-I (AGD was 1.8 days). Our earlier studies[25,31] as well as the results provided here for the MCa-K tumor (Figure 2) showed that apoptosis induction was a main mechanism by which paclitaxel exerted its antitumor action. There existed a high degree of correlation between apoptosis induction and antitumor efficacy of paclitaxel measured by tumor growth delay.[25] There have been a number of reports that paclitaxel can induce TNF activity, apparently mimicking in this respect the activity of lipopolysaccharide (LPS). The drug down-regulates TNF receptors,[32] induces expression of TNF and interleukin-1 mRNA,[41] and triggers release of biologically active TNF.[32] These actions of paclitaxel were either independent[41] or dependent on the presence of LPS.[43] As discussed below, the endogenously released TNF could have participated in the antitumor activity of paclitaxel by making tumor cells more susceptible to paclitaxel-induced apoptosis.

The combination of TNF and paclitaxel produced EFs ranging from 1.24 to 2.17, depending on tumors used. The results obtained using the MCa-K carcinoma suggest that an increased susceptibility of tumor cells to paclitaxel induction of apoptosis was an underlying mechanism for the observed enhancement. Although TNF on its own did not cause apoptosis, it significantly increased apoptotic response to paclitaxel (Figure 6). Also, tumor cells exposed first to paclitaxel showed an enhanced apoptotic response when treated with TNF 1 h later. Therefore, modification of cellular susceptibility to apoptosis induction appears to be a significant mechanism by which TNF enhanced tumor response to paclitaxel.

TNF was the most effective in enhancing paclitaxel's effect against the HCa-I tumor (EF = 2.17) but failed to enhance paclitaxel's effect against the OCa-I tumor (Table 2). HCa-I was the most resistant and OCa-I was the most sensitive of the four tumors to both TNF and paclitaxel when these agents were given as single treatments. The reasons for this difference between HCa-I and OCa-I are unclear. Several reports have shown that TNF and paclitaxel may share a common pathway of signal transduction for apoptosis.[44] Recent studies suggest that cellular microtubules, a major cellular target for paclitaxel, might be involved in TNF production.[45,46] Thus, it is possible that endogenously released TNF after paclitaxel treatment may be one of the mechanisms by which paclitaxel exerts its antitumor effect, and as shown by our data presented here, TNF would make cells more sensitive to apoptosis induction. Based on

this reasoning, a possible explanation for the weak antitumor effect of paclitaxel against HCa-I tumor is that there was a small release of endogenous TNF, and therefore the exogenous TNF was highly effective in enhancing the effect of paclitaxel. The opposite case would occur with OCa-I. Here, the cells were sensitive to paclitaxel due to sufficient release of endogenous TNF; additional TNF could not further increase cellular sensitivity to paclitaxel.

In conclusion, our data show that TNF can be beneficially combined with paclitaxel in the treatment of malignant tumors. The effect was either additive or more than additive; the supraadditive effect was mediated by the increased sensitivity of tumor cells to paclitaxel-induced apoptosis.

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